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Toward Systems Biology in Brown Algae to Explore Acclimation and Adaptation to the Shore Environment

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Abstract

Brown algae belong to a phylogenetic lineage distantly related to land plants and animals. They are almost exclusively found in the intertidal zone, a harsh and frequently changing environment where organisms are submitted to marine and terrestrial constraints. In relation with their unique evolutionary history and their habitat, they feature several peculiarities, including at the level of their primary and secondary metabolism. The establishment of *Ectocarpus siliculosus* as a model organism for brown algae has represented a framework in which several omics techniques have been developed, in particular, to study the response of these organisms to abiotic stresses. With the recent publication of medium to high throughput profiling data, it is now possible to envision integrating observations at the cellular scale to apply systems biology approaches. As a first step, we propose a protocol focusing on integrating heterogeneous knowledge gained on brown algal metabolism. The resulting abstraction of the system will then help understanding how brown algae cope with changes in abiotic parameters within their unique habitat, and to decipher some of the mechanisms underlying their (1) acclimation and (2) adaptation, respectively consequences of (1) the behavior or (2) the topology of the system resulting from the integrative approach.

Introduction

In most marine coasts with significant tidal amplitude, the organisms living in the intertidal zone have to thrive with frequently fluctuating conditions because most of them will be exposed to the air at low tide, and will be underwater at high tide. This dynamic environment, where organisms are periodically submitted to terrestrial and marine constraints, presents different types of habitats, from sandy beaches to steep rocky shores. It is also often impacted by anthropogenic pollution. Within the vegetation adapted to the harsh environmental conditions of the intertidal ecosystem, the dominating brown algae (Phaeophyceae; 1,500–2,000 species) are complex multicellular organisms, with some of them (the kelps) playing a key role as engineer species in benthic flora and fauna assemblages. Most of them are marine and live in temperate and polar water along the coastlines of all continents, even if some kelp forests have been recently discovered in deep-water habitats of tropical regions (Graham et al., 2007; Santelices, 2007), and a few species (less than 1%) can occur in freshwater habitat (McCauley and Wehr, 2007). Brown algae belong to the phylum of stramenopiles (also named heterokonts), a phylogenetic lineage distantly related to terrestrial plants and animals. More specifically, brown algae are part of the Ochrophytes (photosynthetic stramenopiles), for which appearance has been estimated to have occurred 1,000 million years ago (Brown and Sorhannus, 2010). These organisms have arisen after a secondary endosymbiosis, and their current genomic content has also been shaped through evolution by a number of additional lateral gene transfers (Michel et al., 2010a, 2010b). As a result of this complex evolutionary history, combined to a dynamic life environment, brown algae have evolved peculiar features related to basic biological processes, such as primary metabolism and development (Michel et al., 2010a, 2010b; Peters et al., 2008). These algae are also important primary producers (Mann, 1973), and their potential as a source of biomass for the production of bioenergy has recently regained interest.

The study of the acclimation (short-term changes, no genetic control) and adaptation (long-term changes, genetic control) to abiotic conditions has a long history in brown algae, with numerous early studies examining the effect of

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abiotic stressors on algal growth and photosynthesis (reviewed in Soeder et al., 1974), then emphasing on the metabolism of reactive oxygen species (Davison and Pearson, 1996; Dring, 2006). However, all these studies were carried out on a large panel of brown algal species and thus provided relatively scattered information on the mechanisms involved in abiotic stress response and adaptation. In addition, most of the data were physiological, cellular, and biochemical observations conducted at low to medium scale, and there was a lack of knowledge about the genes related to these processes, together with a need for large-scale molecular data to get more insights into acclimation and adaptation in brown algae.

The context has changed a few years ago with the emergence of *Ectocarpus siliculosus* as a new biological model. This small and filamentous brown alga, still a close relative to "giant" kelps species (Silberfeld et al., 2010), has a longstanding research history (Charrier et al., 2008; Coelho et al., 2007; Peters et al., 2004). The recent completion of its genome, the first for a multicellular alga (Cock et al., 2010a, 2010b; for access to the genome, http://bioinformatics.psb.ugent.be/webtools/bogas/), is providing a solid framework in which developing some medium to high-throughput omics techniques, already used for well-established multicellular eukaryotic models. Analysis of the *Ectocarpus* genome has then revealed some insights into potential processes involved in coping with stress in the intertidal zone, such as a complex photosynthetic system, a set of proteins involved in the metabolism of reactive oxygen species, and candidates for halogen-metabolism enzymes (Cock et al., 2010a).

In addition to the report of the *Ectocarpus* genome, a number of recent articles describing transcriptomic, proteomic, and targeted metabolite profiling analyses, performed at medium to high-throughput scales, have been published for this alga, in particular, for studies focusing on the abiotic stress response of this organism (Dittami et al., 2009, 2011a; Ritter et al., 2010). These new datasets represent the groundwork for conducting global integrative analyses in *Ectocarpus*, and then to go further by applying systems biology approaches. So far, such research field has been meaningful mostly in model organisms because it required a high number of the molecular components of the system (such as genes, proteins, and metabolites) to be known. For instance, within eukaryotes, these approaches have been considered to study biological processes such as acclimation and adaptation (analysis of regulatory networks, dissection and modeling of abiotic stress responses/tolerance) of common fungal, animal, and plant models. Among the algae, the new model organism *Ectocarpus* holds great potential for investigating some of the mechanisms underlying these biological processes in the context of a highly variable environment such as the intertidal zone. Systems biology approaches can be applied to study different levels of cell organization (cell, tissue, whole plant, population), and we have chosen to focus this review on analysis at the cellular scale. After giving some insights on recent stress omics data produced in *Ectocarpus*, we describe a gradual approach that will consist in inferring metabolic networks by integrating different levels of knowledge gained in *Ectocarpus* (including genome, transcriptome, and metabolome), and then analyzing dynamic models to infer some of the important aspects related to acclimation and adaptation of the brown algae to their habitat.

Phenotyping Under Abiotic Stress Conditions by Medium to High-Throughput Omics Approaches

Transcriptome profiling

Looking at the changes within the subset of RNA transcripts is probably the most rapid and efficient way to obtain a wide view of the effects of abiotic stress treatments. This was illustrated by construction of cDNA libraries and sequencing of ESTs for two *Fucus* species to study changes in gene expression during aerial exposure, dessication, and heat shock (Pearson et al., 2001, 2010). In parallel to analyses in the fucoid algae, Roeder et al. (2005) have compared EST libraries produced from sporophytes (diploid phase of life cycle) and derived protoplasts (cells from samples where cell wall has been removed by enzymatic degradation) of *Laminaria digitata*, and observed that the latter library was enriched in genes related to mechanisms involved in stress response, such as heat-shock proteins (HSPs), glutathion S-transferases (GSTs), and bromoperoxidases.

With the emergence of *E. siliculosus* as the model species for brown algae, other techniques to perform transcriptomic profiling in these organisms have been then considered. In the study published by Le Bail et al. (2008), dealing with the search for genes that can be used for normalization of gene expression analyses through quantitative real-time PCR analysis, the authors found that two genes were sufficient for normalization of data, and the most appropriate for osmotic stress and chemical treatments were EF1 alpha (EF1α, locus Esi0387_0021 in the *Ectocarpus* genome database) and alpha tubulin (TUA, locus Esi0053_0059). These results were confirmed by microarray data produced 1 year later by Dittami et al. (2009) to monitor changes in gene expression under several conditions of abiotic stresses. This study represents the first large-scale (but not yet fully genome wide) transcriptomic analysis for this new model species, conducted with a custom oligonucleotide array based on EST produced in the framework of the *Ectocarpus* genome project (see details for the array at http://ww.sb-roscoff.fr//UMR7139/ectocarpus/transcriptomics/). In this study, measurement of changes of photosystem II efficiency by pulse modulation fluorometry under different stress conditions, and in comparison with control condition (450 mM NaCl), allowed to select sublethal (i.e., allowing full recovery after stopping the treatment) hyposaline (56 mM NaCl, 6 h), hypersaline (1,470 mM NaCl, 6 h), and oxidative (1 mM H_2O_2, 6 h) stress conditions. From these samples, it has been inferred that 70% of the genes significantly changed expression in one or more of the conditions tested [*t*-test, false discovery rate (FDR) of 10%], featuring a wide reprogramming of the transcriptome under these stresses; among the genes, 67% were unknown, illustrating the high potential for discovery of new stress regulated genes in *Ectocarpus*. One-third of regulated genes left was then analyzed to get insights into the biological processes and metabolic pathways involved in the short-term abiotic stress response. It was observed that primary metabolism was downregulated under these conditions, whereas several pathways related to the use of energy stores and degradation of proteins (autophagy, proteasome) were induced. In addition, some signaling pathways were activated, including several transcription factors and protein kinases representing interesting candidates for further targeted analysis. Interestingly, some classical stress response genes, such

as those encoding proteins involved in scavenging of several ROS species, did not exhibit any activation under the different stresses tested.

A clustering analysis using the same dataset conducted to the establishment of seven different clusters, two of them being enriched in genes encoding chlorophyll binding proteins (CBPs). Further comparison of the *Ectocarpus* stress regulated CBPs, which were shown to be members of the LI818 family, with similar proteins identified in other photosynthetic aquatic and terrestrial organisms and with nonstress CBPs, revealed some important differences between the three-dimensional structure of stress and nonstress CBPs, and also permit to suggest some new hypothesis on the evolution of the LI818 proteins (Dittami et al., 2010). This example illustrates how microarray analyses can, in a new model organism as well, provide relevant figures not only at the global scale, but also for specific proteins that belong to multigenic families.

Proteome profiling

Even if transcriptomic profiling represents a quick way to obtain information genome-wide, all mRNAs may not be translated into proteins. Therefore, availability of reliable protocol to conduct medium- to high-throughput proteomic analysis was necessary for brown algae. In 2008, Contreras et al. reported the establishment of a protocol allowing reproducible production of high quality protein extracts, based on phenol extraction, for two-dimensional gel electrophoresis for the species *E. siliculosus* and *Scytosiphon gracilis*. In the mean time, a similar approach was developed for the Japanese kelp *Ecklonia kurome* (Nagai et al., 2008). In addition, Yotsukura et al. (2010) used an ethanol/phenol extraction method to monitor seasonal variations of protein expression in a different Japanese kelp, *Saccharina japonica*, and observed the increase of production, during the summer, of a vanadium bromoperoxidase, an enzyme known to be involved in stress response in brown algae.

The protocol developed for *Ectocarpus* and *Scytosiphon* was then used to identify proteins regulated by chronic copper stresses. In *Scytosiphon*, differential proteome analysis permitted to select 35 proteins whose corresponding tryptic peptides were further characterized by liquid chromatography (LC) coupled to tandem mass spectroscopy (MS) and by blast analysis (Contreras et al., 2010). Among the proteins overexpressed under copper stress, several were found to be involved in carbohydrate metabolism, active transport, and classical stress response. Ritter et al. (2010) considered two strains of *E. siliculosus*, the reference genome strain (isolated from a nonimpacted site in Peru) and a strain isolated from a copper polluted site in Chile. Assessment of the toxicity of copper in both strains, by a combination of *in vivo* measurement of chlorophyll fluorescence and by epifluorescence microscopy, revealed that the isolate from the contaminated site was less sensitive to copper than the reference strain. Each ecotype was then exposed for 10 days to sublethal copper concentration, 50 μg/L and 50–150 μg/L for the sensitive and tolerant strain, respectively. Proteins extracts from treated and nontreated algae were used for comparative soluble proteome analysis. Differentially expressed proteins between stress and control conditions for each strain, and also between the two isolates, were then identified as previously described for *S. gracilis*. In each strain, copper excess induced the production of proteins involved in different cellular processes such as energy production, glutathione metabolism, as well as accumulation of HSPs. Furthermore, comparison between the two isolates exposed to the same concentration of copper allow identifying features related to copper tolerance in the strain isolated from copper impacted site, in particular, proteins involved in the function and stabilization of the photosystem II, and a vanadium-dependent bromoperoxidase.

Targeted metabolite profiling data integrated with genomic and transcriptomic data

In addition to reports describing changes in cell-wall content (alginates, fucans) and storage polysaccharide (laminarin), mainly conducted to assess the influence of seasonal variation on these compounds (Lobban and Harrison, 1994), there are only a limited number of studies describing targeted profiling of intracellular polar and nonpolar molecules produced through brown algal primary and secondary metabolism. Interestingly, most of them have been performed under abiotic stress conditions.

In 2008, Ritter et al., using a combination of LC-MS and GC (gas chromatography)-MS analysis on samples of *L. digitata* submitted to acute copper stress, reported the increase of release of C18 and C20 polyunsaturated fatty acids and the subsequent enhancement of production of C18 (plant-like) and C20 (animal-like) oxylipins, such as the already known 12-oxo-PDA (phytodienoic acid) and some prostaglandins, but also of a new compound identified as 18-hydroxy-17-oxo-eicosatetraenoic acid. Pharmacological analysis revealed the occurrence of enzymatic and nonenzymatic pathways for the production of a large range of fatty acid oxygenated derivatives. Although fatty acid profiles of brown algae had already been described 10 years ago (see an example in Khotimchemko, 1998), the work of Ritter et al. (2008), which also included an analysis of changes in gene expression of genes potentially involved in stress response, provided some important insights into brown algal molecular copper stress response, in particular, on potential stress signaling molecules. However, a direct link between induction of oxylipin production and regulation of stress genes could not be established. This study was further extended by measuring the release of volatiles aldehydes in air and seawater surrounding copper stressed *L. digitata* (Goulitquer et al., 2009), which reveals that copper enhanced the release of C6 and C9 aldehydes such as hexanal, (E)-non-2-enal, 4-HHE (4-hydroxy hexenal), and 4-HNE (4-hydroxy nonenal). It will now be of interest to decipher more precisely the role of this bouquet of oxylipins in intracellular and interorganism communications.

This pioneer work, however, only dealt with a restricted part of the brown algal metabolism, and did not allow obtaining a more global picture of what can be the changes in the primary metabolism occurring under abiotic stress response. The study on the model species *E. siliculosus* published by Gravot et al. (2010) attempted, by targeting profiling on specific categories of molecules such as amino acids, sugars, polyols, and organic acids, to give a deeper survey on biological processes related to primary metabolism in this organisms. It paved the way for the work of Dittami et al. (2011a), who quantified the changes in the same metabolites, plus the fatty acids, under short-term saline and oxidative

stress conditions already considered for analysis at the transcriptomic level (Dittami et al., 2009). Combination of genomic data, together with transcript and metabolite profiles for the same samples, provides the most integrated view of changes occurring under abiotic stress conditions in brown algae obtained so far. Among the interesting features, the hypersaline stress induced more changes in metabolite contents than the other two tested conditions, and mainly affects amino acid concentrations. Neither urea nor trehalose was quantified, in contrast to mannitol and proline, which accumulate under the saline stress, but at too low concentrations to support their role as compatible solutes at the level of the entire cell. A striking result was the increase of the γ-aminobutyric acid (GABA) content under the hypersaline condition, despite the absence of the genes related to GABA shunt in *Ectocarpus*. The combination of omics data suggest that this rather ubiquitous signal molecule could be synthesized through a salt stress induced putrescine degradation pathway in this alga. Detailed studies are now necessary to confirm or disprove this hypothesis.

From Profiling to Systems Biology Approaches

The data available from the previously mentioned studies allowed depicting the brown alga *Ectocarpus* system at different biological abstraction levels (genome, transcriptome, proteome, and metabolome). Integrating this information is now absolutely required to fully understand how brown algae adapt/acclimate to abiotic factors. As an example, the integration of genomic, transcriptomic, and novel metabolic data emphasized different levels of alteration within several metabolic pathways according to the stress conditions tested (Dittami et al., 2011a). Those observations have confirmed preliminary results (Dittami et al., 2009), and have reinforced evidences of the effects of stresses on primary and secondary metabolism, as well as on other metabolic processes such as photosynthesis. For further investigations, data integration should now be extended; to that purpose, we introduce in the following sections a systems biology protocol for brown algae, and in particular *E. siliculosus*, combining different existing computational approaches.

Considering the current knowledge on brown algal physiology and the inherent complexity of the system, we put forward two distinct assertions: (1) we assume that metabolism represents a major phenotypical scale in brown algae, because impacts of environmental variations at the molecular level so far have been observed mainly on primary and secondary metabolism, and (2) our cornerstone issue is to explain the differential profiles and some of the phenotypical changes observed in several brown algal species under abiotic stress conditions. From a system point of view, such changes are governed either by species adaptation, resulting in changes in the topology of its metabolic networks, or by acclimation, which impacts on the dynamical behaviors of subparts of the system—the so-called functional modules. The computational protocol summarized in Table 1 investigates and discriminates both phenomena. A first step is to build the metabolic networks based on the available *Ectocarpus* genome. Then, the reconstructed metabolism is compared with additional metabolic pathways information (from brown algae when available, or benchmark plant metabolisms for instance when relevant). In a third step, both environmental information and

Table 1. Gradual Bioinformatic Analysis to Study Acclimation and Adaptation in Brown Algae by a Systems Biology Approach

	Data and knowledge	*Goals*	*Methods*	*Biological applications and interpretations*	
1	Literature knowledge + brown algal genomes + chemical database	Metabolic networks reconstruction	Pathway Tools + completion by homology, *ab initio* and manual analysis	Synthesis of a **metabolism** from a given genome	
2	**Metabolisms**[a] from two distinct species	Metabolism comparison	graph comparison	Identification of specific reactions within species (topology of the networks)	ADAPTATION
	Metabolisms[a] from two distinct species	Metabolic pathways comparison	Elementary flux modes extraction	Identification of pathways/crossroads of reactions that are specific of species	
3	**Metabolisms**[a] from two distinct species + environmental conditions	Flux within pathways	Flux Balance Analysis	Identification of pathways used in given environmental conditions	ACCLIMATION
	Metabolism[b] + transcriptomic data from different conditions	Functional modules	Graph approach (k-SIP)	Identification of proteins involved in metabolic pathways under distinct conditions, of functional **modules** and their potential regulators	
4	**Modules** + transcriptomic data + physiological knowledge	Connected modules	Automatic reasoning and numerical simulations	Automatic building of numerical models, mechanistic explanation of biological behavior (acclimation and/or adaptation)	

[a]One compares the topologies of metabolic network. For this task, two distinct metabolisms must be considered.
[b]A single metabolism is needed here.

transcriptomic results can be integrated to quantify the impact of adaptation and/or acclimation in an automatic manner. Once the two biological hypotheses have been tested, a final step should allow performing automatic reasoning over the system to identify the regulatory mechanisms underlying response to abiotic cues. These different steps are described in more details in the subsequent paragraphs.

Building the metabolic networks from omics data

Reconstruction of metabolic networks is a difficult and error-prone task. It involves mapping genes from the target species to enzymatic functions, and using those functions to predict which biochemical reactions exist in the metabolism of the species of interest. As complementary knowledge, numerous public resources such as KEGG (Kanehisa and Goto, 2000), and BioCyc (Karp and Caspi, 2011), are available. They can be integrated in tools like Pathway Tools (Karp et al., 2010). Such standard techniques have shown great results when applied to bacterial species or benchmark eukaryotic species, among which *Arabidopsis thaliana* (Poolman et al., 2009). When applied to underinvestigated species, these tools based on genome data may quickly reach their limits, unless taking into account information other than genomic sequences, as achieved for *Chlamydomonas reinhardtii* by integrating several molecular repertoires via greedy algorithms (May et al., 2008). In particular, several specific metabolic reactions can be missing in the commonly used metabolic pathway databases, thus affecting automatic reconstruction solely based on genome information (Pitkänen et al., 2010; Ruppin et al., 2010). To overcome this situation, metabolite profiles and prior biochemical knowledge about the organisms of interest can be used to refine this mapping (Ng et al., 2006). More specifically, transport reactions or intracellular localization of enzymes, which represent key processes for investigating phenotypical changes under environmental constraints, are often missing or are only partially considered in public databases. Boyle and Morgan (2009) overcame this problem in the green microalga *Chlamydomonas reinhardtii* by focusing the reconstruction on known metabolic reactions that are responsible for phenotypical behaviors. To do so, the authors anchored the core of the metabolic network to the fatty acid metabolism, well characterized at the biochemical level in this organism, and which represent also a cornerstone within the global metabolism under several growth conditions. In addition, they assumed enzymatic reactions to be reversible if no information was available. Other metabolic reconstructions have been performed following similar trends with great successes, for instance, in several land plants (Dal'Molin et al., 2010a, 2010b; Saha et al., 2011; Urbanczyk-Wochniak and Sumner, 2007; Zhang et al., 2010). In the context of brown algae, the mannitol metabolism, which has a central role in the physiology of these organisms and for which molecular data have been recently published (Michel et al., 2010a; Rousvoal et al., 2011), should be considered as the core biological information to use for anchoring the *Ectocarpus* metabolic network. Building a network centered on these reactions is a way to attest that the metabolism, as reconstructed, will be appropriate to analyze phenotypical changes of brown algae under different abiotic treatments.

As a major weakness, the efficiency of this task remains closely dependent of the quality of the genome annotation. To avoid potential mistakes, it is relevant to compare the resulting metabolic networks with *ab initio* reconstructions, as suggested by Boyer and Viari (2003) and Heath et al. (2010). These graph theoretic techniques have been developed to find optimal ways to go from a given substrate to a given product by tracking the atoms that are involved in metabolic reactions. This type of reconstruction features the succession of reactions that minimize the number of transferred atoms potentially used for the completion of metabolism. Despite their computational complexity, these approaches remain suitable for investigating and validating previously uncharacterized metabolic pathways, in particular, when critical parts of the metabolism have been clearly identified, like the mannitol cycle for the brown algae.

Investigating the metabolic network topology

Once metabolic network has been checked by taking into account biological knowledge of the species of interest, it has to be compared with previously known metabolisms from different organisms. It is implicit herein that, when conducting the comparison of metabolism (presence or absence of a given metabolic reaction), it is important to put the emphasis on selecting genes that only encode enzymes. Despite this restrictive assumption, metabolism comparison should be applied to metabolic pathways of interest and other benchmark metabolisms. In the case of brown algae, inspection of metabolisms between different species remains an interesting prospective application when several genomes will be available.

The comparison of metabolism encompasses two aspects. The first focuses on the metabolic network topologies that can be defined as oriented graphs. From a combinatorial point of view, comparing these graphs is a difficult and a complex task. Nevertheless, in the context of the brown algae, metabolic reactions are well identified by their tags (tags defined by Pathway Tools; Prigent et al., unpublished results), which give the opportunity to apply standard graph comparison techniques (Yamada and Bork, 2009). Among the ones available, Mano et al. (2010) described a computational approach to determine a distance measure between metabolic graphs, based on the topology of the network. By showing clusters of metabolic pathways (i.e., set of reactions connected to each other by sharing common metabolites) that are conserved over distinct species, it indicates pathways that have similar evolution. Even if this technique has not been yet applied to brown algae, the fact that a similar approach showed accurate results when reconstructing phylogeny (Chang et al., 2011) indicates a promising application for these organisms.

The second aspect relates to the comparison of crossroads of reactions. Effectively, a metabolism can be considered as a network of molecules and as the enzymatic transformations altering the contents of these molecules. Such a representation highlights the multiple pathways that compounds can undergo to enter and leave the metabolic system. Within the network, crossroads of pathways represent important reactions due to their potential impact on the fate of many substrates. Several techniques, mainly based on decomposition of the fluxes going through the metabolic network into a set of elementary flux modes, allow to analyze the metabolic flux of a balanced metabolic system (Gagneur and Klamt, 2004; Rezola et al., 2011; Schuster et al., 2000; Terzer and Stelling,

2008). This description of metabolism is minimal and each elementary mode represents a metabolic process (i.e., substrate to product transformation). By definition, the linear combination of all the modes accounts for the whole set of transformations that passes through the metabolism. In particular, Christian et al. (2009) proposed a coupling of flux balance analysis with the hidden Markov Model (HMM) to correct and trim the *Chlamydomonas reinhardtii* reconstructed network, considering also that stoichiometric knowledge was available. Beyond the minimal description of the metabolism, such decomposition of the network emphasizes the crossroads of modes (i.e., reaction shared between several modes). Because modifying these crossroads potentially impacts the connected modes, the specific reactions and their corresponding crossroad genes are essential (Cornish-Bowden and Cadenas, 2000). The number of essential reactions or genes is an abstraction of the system robustness, and describes the inherent modularity of the metabolism (Kitano, 2004). The more the system includes essential genes, the more the metabolic modes are connected, supporting the potential capacity of the system to switch the metabolic flux from one mode to another when being challenged. In addition, the decomposition will indicate the elementary modes that should be impacted by a given environmental stress: a treatment altering a metabolic input will impact the whole set of elementary modes that use this input as substrate. Application of this technique to stressed brown algae should permit to determine the signaling and/or metabolic cascades involved in physiological response, and therefore give some insights into mechanisms underlying acclimation and adaptation. In a mid- or long-term perspective, with more brown algal genomes available, it will be possible to compare the effect of similar abiotic stresses on the metabolism of different species. In addition, this approach, if applied on two distinct phyla, might show similar impacts (i.e., similar modes impacted) despite the phylogenic distance.

Integrating complementary knowledge to distinguish adaptation from acclimation impacts

Once the algal metabolism is described by its topology and its ability to respond to a stress, it will be possible to integrate complementary knowledge/data to estimate if and how metabolic behavior tuning is a consequence of adaptation or acclimation.

To do so, a first approach consists in incorporating environmental conditions. A natural extension of the modular decomposition of metabolic reaction networks into elementary flux modes is to estimate the flux that pass through the modes at steady state using a mathematical method called the flux balance analysis (FBA). To this aim, environmental conditions should be considered as a set of quantities of substrates and products. When applying this information, and knowing the relative stoichiometry of the metabolic reactions, it is possible to perform a linear optimization to quantify the distribution of the flux within the system at equilibrium that maximize the mannitol production. Indeed, for brown algae, the values of mannitol content can represent a major parameter to be considered to study the algal adaptation. This FBA technique has already demonstrated its efficiency (Boyle and Morgan, 2009), and can also be used to classify the reactions (or corresponding genes) that are the most affected by variations of environmental conditions (Papp et al., 2004). This approach should then be taken into account as guidance for future experiments on target enzymes. Moreover, it should also be used to study the metabolic behavior of brown algae under distinct environmental conditions. Changes occurring in the behavior of the metabolism will thus be related to acclimation process rather than adaptation.

A second approach is based on the integration of results obtained at the transcriptomic level. Effectively, gene expression profiling data allow studying genes that are coexpressed under distinct conditions. A multivariate analysis based on the correlation between genes discriminates clusters of genes that follow the same pattern of variations. Analysis of genes that are coexpressed can be performed as described by Stuart et al. (2003), and enrichment of functional categories within clusters can be examined by Gene Ontology (GO) (Boyle et al., 2004).

In addition, when time-series data are available, including profiling at different levels of molecular organization, and their covariance stationarity certified, it is possible to apply the concept of Granger causality (for description. see Lozano et al., 2009) to investigate possible temporal hierarchy and mutual relationships between genes and other types of molecular actors. For instance, using this approach showed great results for yeast exposed to heat and cold stress, by indicating that temporal behavior was consistent with cause-effect associations (Walther et al., 2010).

To complement these methods, and as an alternative to the previously described FBA, we suggest a graph theory based approach that combine metabolic networks with transcriptomic datasets produced under different experiments, as previously depicted in benchmark prokaryotic and eukaryotic systems biology models (Bordron et al., 2011a, 2011b; Ihmels et al., 2004, Wei et al., 2006). Because the metabolic network corresponds to a graph, an edge in this representation can be considered as a link between two reactions for which the product of one is the substrate of the other. Providing edges with a measure, such as the correlation between genes corresponding to two successive enzymatic steps, we transform the metabolic network into a weighted graph. Thus, from given substrate and product, extracting the pathways that maximize the sum of weights between sequential reactions emphasizes a metabolic pathway for which the corresponding genes are mostly coexpressed. By extension, when applied to the whole metabolic network and by a full set of transcriptomic data, it highlights the genes encoding proteins catalyzing successive enzymatic steps and that are coexpressed under a distinct condition. These sets of genes, characterized either by the metabolic pathways they belong to, and by their coexpression values, are called "modules," which can be considered as functional under a stress condition (i.e., expressed simultaneously and belonging to a metabolic pathway). The behavior of the network induced by these selected modules is thus explained by the adaptation (topology of the metabolic network) or the acclimation (changes in the transcriptome). Application of this technique to brown algal systems will allow comparing transcriptional behaviors under different conditions in order to estimate if alterations of algal physiology are mainly explained by adaptation of the metabolism or by metabolic changes related to acclimation. Generalization of this approach or related techniques to visualize the integration of these data (Droste et al.

2011) will be a guidance to predict the metabolic behavior in various brown algal ecotypes.

Towards numerical models

The integrative approach described in the previous paragraphs highlights the set of metabolic reactions that are consistent both at the metabolic (i.e., metabolic cascade) and transcriptomic levels. To a certain extent, the same approach could be used to explain the function of genes encoding proteins other than enzymes (called below nonenzymatic genes). By focusing on enzymatic genes, our integrative approach, as mentioned above, provides detailed information about the metabolic pathways involved in clusters of genes discriminated by transcriptomic analysis. To go further, we can consider that the nonenzymatic genes, that were not taken into account so far, are potential regulators of the highlighted pathways or functional modules. Considering transcriptomic datasets produced for different conditions, two distinct functional modules can be connected if they are under the influence of the same regulator(s). By extension, this identification connects modules to each other according to the use of their shared regulators (i.e., regulators are condition specific in function of the modules), producing a network of modules. The activation of a module instead of another (via its regulators) reflects an impact of the acclimation effect, at the transcriptomic scale. Extending this sketch of reasoning to the whole brown algal system could be difficult. Automatic constraints based techniques like global reasoning (Baumuratova et al., 2010; Guziolowski et al., 2009; Veber et al., 2008) can be used for investigating sketch of regulatory networks as depicted here. It should allow deciding which regulators in the network of modules need to be present or absent to globally explain activation of modules. In the context of algae, transcriptomic information, because they represent an abstraction of the metabolic network, can be used as global constraints connecting edges of the network of functional modules, to eventually complete a partial algal metabolism. Note herein that other techniques have been suggested to investigate metabolic networks by using graph theory based approaches (Cottret et al., 2010). As a result, automatic reasoning may also isolate subparts of the metabolic network that behave differentially under environmental constraints. These subparts consist in connected modules. After this reduction, it should be possible to investigate the dynamics of this connection of modules with numerical modeling to produce quantitative or kinetic models. As an illustration, this approach may be useful to identify components that regulate the mannitol metabolism or other functional modules that can be considered as essential to control the brown algal physiology. From a modeling point of view, one can also consider this last step as an automatic way to produce, from omics data, ordinary differential equation models, as commonly used in plant physiology modeling.

Conclusions and Perspectives

Significant advances in the description of the molecular abiotic stress response have been made in the last years for brown algae, and in particular for *Ectocarpus*. However, omics approaches are still in their infancy for this alga, and it is important to mention that most of the work published so far has focused on the acclimation (short-term changes) in response to *in vitro* alterations, and analyses have been carried out with the *Ectocarpus* reference genome strain. It will now be interesting to exploit the diversity of the *Ectocarpus* ecoytypes, with more than 300 strains available at the CCAP collection (Gachon et al., 2007; http://www.ccap.ac.uk/). These ecotypes should allow studying the adaptation to specific environmental conditions, and also their acclimation when placing them under conditions different from their habitat of origin. Typical examples to be considered are the freshwater strain (West and Craft, 1996), and the strain tolerant to copper (Ritter et al., 2010). Analysis of these ecotypes by different omics approaches, including sequencing of their genomes, should provide strong support for comparative analysis and insights into the mechanisms driving adaptation of these strains to diverse aquatic conditions. Studies on these ecotypes is also relevant in the actual context where species concept is changing in *Ectocarpus* (Dittami et al., 2011b; Peters et al., 2010a, 2010b), with this genus possibly containing more species than it is currently acknowledged. In parallel, high-throughput sequencing of new *Ectocarpus*, and hopefully other brown algal genomes, represents a method of choice to detect small noncoding RNAs (including miRNAs and antisense transcript RNAs), in relation with the importance of these molecules in the regulation of some mechanisms underlying abiotic stress response.

In term of regulation, a lot is happening at the transriptomic level, but metabolic processes may also be regulated at post-transcriptionnal and posttranslational level. Modeling of omics data should help deciphering at what level important changes occur. In addition, systems biology approach should allow generating some hypothesis, which could be tested by combining targeted analyses at the biochemical and genetic level to go deeper in the understanding of the mechanisms of stress response. There are a few examples of functional characterization of enzymes from *Ectocarpus*: GSTs (de Franco et al., 2009), mannitol-1-phosphate dehydrogenase (Rousvoal et al., 2011), GDP-mannose dehydrogenase (Tenhaken et al., 2011). In parallel, forward and reverse genetics approaches are needed to characterize some of the molecular actors involved in abiotic stress reponse. The development of targeted genetic analysis could take advantage of a library of mutants generated using a Targeting Induced Local Lesions IN Genomes (TILLING) approach, which is currently in progress for *Ectocarpus*. We do not have access yet to a protocol allowing altering, even transiently, the expression of genes of interest in this alga. Similarly, no protocol is available for genetic transformation of this organism, and these techniques are badly needed. Despite this situation, first reports on the characterization of natural (Peters et al., 2008) and artificial mutants (Coelho et al., 2011; Lebail et al., 2010, 2011), altered in their development pattern, have been published. The availability of a genetic map generated recently for the *Ectocarpus* reference strain (Heesch et al., 2010) represents a valuable tool for further genetic analysis.

Once protocols are established and validated for integrating heterogenous knowledge and for proposing regulatory networks linked to abiotic stress response in *Ectocarpus*, it will be possible to expand the approach to other biological issues and to search for connections between different pathways and processes. Among tracks of interest, which will need the production of other datasets to be studied, one of them is exploring the putative crosstalks occurring between abiotic and biotic stress responses. In particular, despite the fact that

little is known so far on signaling in brown algae, first results indicate that these organisms have conserved the ability to produce a high diversity of molecules known to be involved in signaling in plants and animals, which suggest the occurrence of novel modes of regulation. One of the other aspects to consider can be the relationships between acclimation/adaptation and morphology/development, including for instance investigation of a possible link between the different stages of the life cycle of the alga and ability to cope with highly changing environmental conditions.

To finish, it is important to mention that an *Ectocarpus* database will be necessary in the near future to compile observations carried out at different levels of cell organization and in relation with important biological processes, such as development and stress. This should increase awareness around this new biological model and potential for identifying novel features.

Final Remarks

Fifteen years ago, Davison and Pearson (1996) stated "One major area where progress is needed is in the application of modern molecular, biochemical and physiological techniques, not only to allow us to understand how algae tolerate tidal emersion but to provide diagnostic probes that can be used to measure occurrence of stress in the natural environment. There are opportunities for large numbers of innovative, exciting, and rewarding research projects. Hopefully, it will be possible for us to write another review on this topic in twenty five years time and face the problem of a surfeit, rather than a deficit, of information." The availability of the *Ectocarpus* genome and development of omics techniques, followed by integration of molecular datasets and systems biology approaches, is placing study on acclimation and adaptation in brown algae at the forefront of research in abiotic stress, and there are yet 10 years to go to keep on developing knowledge and to transfer it into the field.

Author Disclosure Statement

The authors declare that no competing financial interests exist.

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